HAYATI Journal of Biosciences, December 2008, p 145-154 Vol. 15, No. 4
ISSN: 1978-3019

Molecular Characterization of Indonesian Indigenous Chickens based on Mitochondrial DNA Displacement (D)-loop Sequences

SRI SULANDARI[1]*, MOCH SYAMSUL ARIFIN ZEIN[1], TIKE SARTIKA[2]

[1]Research Center for Biology-LIPI; [2]Centre for Animal Research and Development, Widyasatwaloka Building, Cibinong Science Center, Jalan Raya Jakarta Bogor Km. 46, Cibinong 16911, Indonesia

Received April 11, 2008/Accepted November 7, 2008

The Mitochondrial DNA (mtDNA) displacement (D)-loop sequences were used to study the genetic diversity and relationship of Indonesian indigenous chickens. A total of 483 individuals belonging to 15 population breeds and 43 individuals belonging to 6 populations of jungle fowl (2 populations of *Gallus gallus* and 4 populations of *Gallus varius*) were sampled. The hypervariable I (HVI) segment of the D-loop was PCR amplified and subsequently sequenced. The sequences of the first 397 nucleotides were used for analysis. Sixty nine haplotypes were identified from 54 polymorphic sites with polymorphism between nucleotides 167 and 397 contributing to 94.5% of the sequence variation. Phylogenetic analysis indicates that Indonesian indigenous chickens can be grouped into five distinct clades (clade I, II, IIIc, IIId, and IV) of the previously identified seven clades (clade I, II, IIIa, IIIb, IIIc, IIId, and IV) in Asian indigenous chickens. Fifty haplotypes belong to clade II, seven haplotypes are in clade IV, six are in clade IIId, three are in clade I and one haploype is in clade IIIc. There was no breed-specific clade. Analysis of Molecular Variance (AMOVA) based on partial D-loop sequences of Indonesian chicken indicates that 67.85% of the total sequence variation between haplotypes was present within the population and 32.15% between populations. One of the haplotypes (represented by PLC4) was shared by all populations, suggesting that these populations may share the same maternal ancestor. These results show a high mitochondrial D-loop diversity and indicate multiple maternal origins for Indonesian indigenous chickens.

Key words: Indonesian indigenous chicken, mitochondrial DNA, D-loop, haplotype, phylogenetic analysis and clade

INTRODUCTION

Chickens are classified as order: Galliformes, family: Phasianidae and genus: *Gallus* (jungle fowl). Domestication resulted in basic changes in the behaviour, physiology and production of the bird, but still there are some similarities between the ancestor and the current chickens (Al-Nasser *et al*. 2007). It is widely believed that all populations of domesticated chicken descend from a single ancestor, the red jungle fowl (*Gallus gallus gallus*), which originated in Southeast Asia (Fumihito *et al*. 1994, 1996). Chicken is by far the most widely distributed livestock. In Indonesia, domestic (indigenous) chicken scattered throughout the archipelago apparently has a lot of diversity with different morphologic characteristics. From 31 breeds of indigenous chickens identified (Nataamijaya 1996, 2000), 11 breeds of chicken are known as chickens with high egg production (free-range/ Kampung chicken, Sentul chicken, Wareng chicken, Black Kedu chicken, Sedayu chicken, Nusa Penida chicken, Merawang/Merawas chicken, Sumatra chicken, Pelung chicken, female Gaok chicken, and Dupin chicken). Twelve breeds of chicken are known as ornamental chickens because of their voice; they are also known as fighting cocks and regarded as chickens with power (Pelung chicken, Ciparage chicken, Banten chicken, Black Kedu chicken, Cemani chicken, Olagan chicken, Kokok Balengek chicken, male Gaok chicken, Tolaki chicken, Bangkok chicken, Bekisar chicken, and Balinese chicken). Four breeds of chicken are known as broilers (free-range/Kampung chicken, Lamda chicken, Nagrak chicken, Black Kedu chicken), but there are also 9 breeds of chicken which superiority has not yet been discovered (Walik chicken, Siem chicken, White Kedu chicken, Maleo chicken, Jepun chicken, Ayunai chicken, Tukung chicken, Burgo chicken, and Nunukan chicken).

Nevertheless, the genetic potential in almost all chicken breeds has not yet been much revealed. As revealed from studies (Nishida *et al*. 1982; Mansjoer *et al*. 1989; Hashiguchi *et al*. 1993; Mansjoer *et al*. 1996; Putra 1999; Kusuma 2002) conducted in the period of 20 years, apparently only around 25% of Indonesian indigenous chicken breeds had been used for research activities, amongst all were Pelung chicken, Sentul chicken, Kedu chicken, Merawang chicken, Cemani chicken, and Kampung chicken. Knowledge on the distribution of chicken genetic diversity in Indonesia would be useful in optimizing both conservation and utilization strategies for indigenous chicken genetic resources. In the past, attempts had been made to characterize local chickens using morphological traits (such as plumage colour, feathering pattern, etc.) which have limited utility in the study of genetic variation. Beside that, the criteria, which was carefully studied on the point of morphology for character monitoring, both qualitative and quantitative, the subjective factorsubjectivity feels dominant in the study result and information gained through the study lacks accuracy. The use of DNA technology by PCR-RFLP technique on D-loop mitochondrial part has also been done on Kampung chicken from some locations to base the selection program. So far, Sartika *et al*. (2000, 2004)

*Corresponding author. Phone: +62-21-8765056, Fax: +62-21-8765068, E-mail: ssulanda@yahoo.co.id

had used microsatelite as the genetic marker to learn the genetic variety on four breeds of Indonesian indigenous chickens (Kampung, Pelung, Sentul, and Black Kedu).

Molecular technology development at the moment has increased the efficiency and accuracy in the genetic characterization study among breeds of animals. A survey conducted by Baumung *et al*. (2004) in 87 researchers (50% from respondents being surveyed) throughout the world shows that DNA is the genetic feature mostly used for genetic characterization in almost all indigenous cattle. Lately, the genetic variety study seen from mitochondria DNA (mtDNA) has very much developed since mtDNA has a high copy number of (10^3-10^4 copies) mollecule mtDNA/somatic cell. The small size of mtDNA can be learnt as a whole. Genom mtDNA has an evolution rate of 5-10 times faster compared to nuclear genome (Brown *et al*. 1982) and mtDNA sequences have been successfully used to determine genetic diversity in Asian chicken (Niu *et al*. 2002; Liu *et al*. 2004). Komiyama *et al*. (2003, 2004a,b) has also used mtDNA to investigate the origin of Japanese gamecocks and their evolutionary relationship with long crowing chickens. Different regions of the mtDNA evolve at different rates.

The area Displacement-loop (D-loop) has alkali variation which is quite high, more polimorphic compared to other area mtDNA (Quinn & Wilson 1993; Ishida *et al*. 1994). As statement revealed by Brown *et al*. (1982), Quinn dan Wilson (1993), Fumihito *et al*. (1994), D-loop area is often used for phylogenetic analysis, both inside the species, and among species. MtDNA D-loop sequences have also been used to describe variation in wild ancestors and domesticated breeds. Comparison of the D-loop sequences of ancestor and domestic populations has given insight on the timing and location of domestication events that produced the livestock of today (Bruford *et al*. 2003). For instance, analyses of mtDNA D-loop sequences were used to investigate the origins of cattle types found today. There is evidence from the mtDNA D-loop variations in European, African, and Indian cattle breeds that indicate independent domestications of *Bos taurus* and *Bos indicus* cattle in two separate locations (Loftus *et al*. 1994). D-loop sequences have also been used in unravelling domestication and diversity of dog (Savolainen *et al*. 2002), horse (Jansen *et al*. 2002), and goat (Luikart *et al*. 2001) to name but a few. Currently the two most popular classes of markers in livestock genetic characterization studies are mtDNA sequences particularly the sequence of the hypervariable region of the D-loop or control region, and autosomal microsatellite loci (Sunnucks 2000). The D-loop has a central domain, which is conserved, and hypervariable segments flanking it on either side. This control region serves as a point of initiation for the replication of mtDNA.

Based on the findings above, the hypervariable 1 (HV 1) D-loop mtDNA sequence was used in this study to reveal the genetic diversity, determine distinctiveness and the phylogenic relationship of 15 Indonesian indigenous chicken breeds (Cemani, Kedu, White Kedu, Merawang, Kapas, Kate, Arab Goden, Arab Silver, Sentul, Pelung, Wareng, Gaok, Nunukan, Tolaki, and Kalosi).

MATERIALS AND METHODS

Blood Sample Collection. Blood samples from 483 indigenous chickens belonging to 15 breeds (Cemani, Kedu, White Kedu, Merawang, Kapas, Kate, Arab Goden, Arab Silver, Sentul, Pelung, Wareng, Gaok, Nunukan, Tolaki, and Kalosi) were used as DNA materials in this study (Table 1 & Figure 2). The samples were transported to the genetic laboratory of Zoology Division, Research Center for Biology - LIPI for analyisis.

Amplification of Mitochodrial DNA and D-loop DNA Sequence. DNA was extracted from whole blood using phenol/chloroform procedure (Sambrook *et al*. 1989) and precipitated with ethanol. To amplify the mtDNA D-loop HV1 region, specific primers based on the published mtDNA sequence were used. The primers were L16750 (5'-aggactacggcttgaaaa gc -3') as forward primer and CR1b (5'-ccatacacgcaaaccgtctc -3) as reverse primer. The gene sequences are based on the partial chicken mitochondrial genome of Komiyama *et al*. (2003) (GenBank accession number AB098668) and complete chicken mitochondrial genome of Desjardins and Morais (1990) (GenBank accession number NC 001323).

PCR reactions were performed in a 30 µl reaction containing 2.5 mM of each dNTPs, 14 pmol of each primer, 1.5 mM MgCl2, 1 × PCR buffer comprising of 10 mM Tris-HCl (pH 8.3) and 50 mM KCl, 1.25 U Taq DNA polymerase, 40 ng DNA template and dH_2O. Reactions of PCR were made in 0.2 ml tubes and the reaction process was carried out on the thermocycler machine Gene Amp*PCR system 9700 (Applied Biosystem, USA). The PCR condition used was Pre Denaturation at 94 °C for 5 minutes, continued with 35 cycles at 94 °C for 45 second, 60 °C for 45 second and 72 °C for 90 second. The last cycle was followed by a temperature of 72 °C for 10 minutes.

Table 1. Blood Sample Collection of Indonesian Indigenous Chicken Breeds

Chicken breeds	ID name	Site collection	Total samples
Cemani	CMP	BPTU Ayam, Sembawa, South Sumatera	2
	CM	Kedu, Temanggung, Central Java	35
Kapas	KPS	Kedu, Temanggung, Central Java	30
Pelung	PL	BPTU Ayam, Sembawa, South Sumatera	20
	PLC	Cianjur, West Java	30
Arab Golden	ARG	Kedu, Temanggung, Central Java	30
Merawang	MR	BPTU Ayam, Sembawa, South Sumatera	30
Arab Silver	ARS	BPTU Ayam, Sembawa, South Sumatera	30
Kedu	KD	Kedu, Temanggung, Central Java	30
	KDH	Kedu, Temanggung, Central Java	12
Kedu Putih	KDP	Kedu, Temanggung, Central Java	18
	KDPJ	Jatiwangi, West Java	9
Kate	KT	Yogyakarta, DIY	31
Gaok	GA	Bangkalan, Madura Island	10
Sentul	STJ	Jatiwangi, West Java	31
	STC	Ciamis, West Java	17
Wareng	T	Tangerang, Banten	12
Tolaki	KTO	Konawe, Southeast Sulawesi	21
Kalosi	KAL	Gowa, South Sulawesi	30
Nunukan	NT	Tarakan, East Kalimantan	30
	NN	Nunukan, East Kalimantan	13
	NS	Sebatik, East Kalimantan	12
Total samples			483

```
          0011111122222222222222222222223333333333333333333
          2366779911222222233344445667889900001112344556669 9
          9178173927012589378236761800126268903520274503716

Ref       CATCTACTATTCACCCCAGGTTATCATTAATCTCTCCTTCAATTTCTCT
KDH2      ........G..........CC.CTG..G.C.C..T.C...........
KTO11     ........G......T...CC.CT...G.C.C..T.C...........
KAL15     ........G..........CC.CT...G.C.C..TTC...........
KDH4      ........G..........CC.CT...G.C.C..T.C...........
NN4       ........G.......T..CC.CT...G.C.C..T.C...........
KTO21     ........G..........CC.CT...G.C.C....C...........
KAL2      ........G............C.CT...G.C.C..T.C...........
KTO4      ...T.T..G..........CC.CT.....C.C..T.C...........
KTO1      ...T.T..G..........CC.CT...G.C.C..T.C...........
KTO10     ........G..........CC.CT..CG.C.C..T.C...........
PLC9      ........G..........CC.CT...G.C.C.CT.C..G........
PLC23     .T......G..........CC.CT...G.C.C.CT.C..G........
KP83      ........G..........CC.CT...G.C.C..T.C..G........
N88       ........G..........CC.CT...G.C.C..T.C..G.C......
NT11      ........G..........ACC.CT...G.C.C..T.C..G........
N812      ........G.C........CC.CT...G.C.C..T.C..G........
AR826     ........G.C........CC.CT...G.C.C.CT.C..G........
NT13      ........G.C........CC.CT...G.C.C..T.C...........
KT19      .......CG..........CC.CT...G.C.C..T.C...........
CMP1      .......CG..........CC.CT...G.C.C..T.C..G........
CM31      T.......G..........CC.CT...G.C.C.CT.C...........
KDP7      T.......G..........CC.CT.C.G.C.C.CT.C...........
KD4       ........G..........CC.CT.C.G.C.C.CT.C...........
NT24      ........G..........CC.CT.C.G.C.CT.T.C...........
PLC1      ........G..........CC.CT...G...C..T.C.........T.
KDP12     ........G..........CC.CT..CG...CT.T.C.........T.
CM5       ........G....T.....CC.CT...G.C.C..T.C.........T.
CM29      ........G..........CC.CT...G.C.C..T.C.........T.
NN1       ........G.C........CC.CT...G.C.C.CT.C.........T.
PL11      ........G..........CC.CT...G.C.C.CT.C..G......T.
KT28      ........G..........CC.CT...G.C.C.CT.C.........A.
PLC20     ........G..T.......CC.CT...G.C.C.CT.C...........
T1        ........G..........CC.CT...G.C.C.CT.C...G.......
GA4       ........G..........CC.CT...G.C.C.CT.C...........
KAL6      ........G..........CC.CT...G.C.C.CT.CC..........
STJ28     ........G..........CC..T...G.C.C.CT.C...........
MR7       ........G..........CC.CT...G...C.CT.C...........
PLC8      ........G..........CC.CT...G.CTC.CT.C...........
KD9       ........G..........CC.CT...G.CTC.CT.C.....C.....
STJ2      ........G..........C..CT...G.CTC.CT.C...........
STJ15     ........G..........C.TCT...G.C.C.CT.C...........
KAL7      ........G..........C..CT...G.C.C.CT.C...........
STC14     ........G.........A.C..C....G.C.C.CT.C...........
GA3       ........G..........CC.C....G.C.C.CT.C...........
KDP2      ........G..........CC.C....G.C.C..T.C...........
KT8       ........G..........CC.CT...G.C...CT.C...........
T2        ........GC.........CC.CT.....C....T.C...........
AR85      ......A.GC.........CC.CT.....C....T.C...........
T7        ........GC..G......CC.CT.....C....T.C.T.........
KP84      ........GC......T...CC.CT.....C....T.C...........
TW2       ........GC...........C.CT.....C....T.C...........
KD13      .......CGC.........CC.CT.....C....T.C...........
KP829     ........GC.........CC.CT.....C....T.C..G........
KT18      ........G..........ACC.C....G.C....T.C..G...CTC..
NT10      ........G..........ACC.C....G.C....T.C..G....TC..
CM33      ........G..........ACC.C....G.C..T.T.C.......TC..
CM10      ..............T..G..C..C....G.C....T.C.....C.T.T.
STC12     ....C..............CC.CT.........T.CA...C..T.T.
KD3       ...............................................C
KP88      ..............................C................C
KD10      ..............................C................
KT7       ............................GC................
ARG2      ..............................C........T........
ARG26     ..............................C.............T.
STC16     ..C.....G....T.......C.........C......C.........
```

Figure 1. Nucleotide polymorphisms observed in D-loop domain of 434 chicken sequences. Vertically oriented numbers indicate the site position and the sequences shown are only the variable sites. Dots (.) indicate identity with the reference sequence (GenBank accession number AB098668) (Komiyama *et al*. 2003), different base letters denote substitution while dashes (-) refer to insertion or deletion.

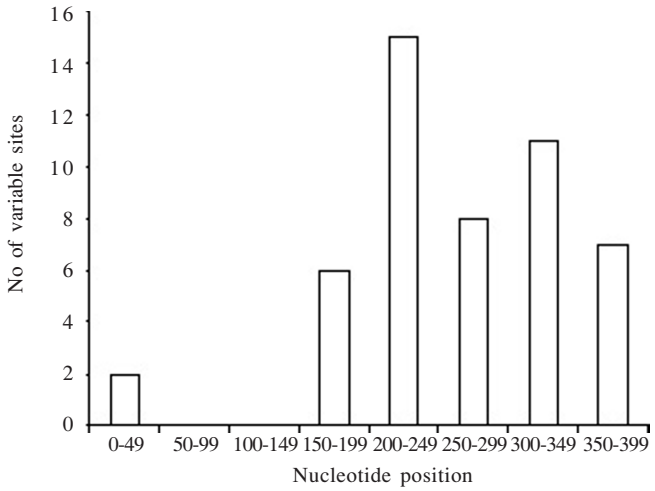

Figure 2. Sequence of variance distribution on segmen HV I from D-loop DNA mitochondria of Indonesian indigenous chicken.

Visualization of the PCR product was conducted by electrophoresis with 2% agarose gel (w/v) stained with ethidium bromide in a 1 X TAE buffer at 100 Volts for 30 minutes. The PCR products were purified using QIAquick kit purification PCR (Qiagen, GmbH, Germany). The purified products were then sequenced using machinery DNA sequencer to find traces of the nucleotide sequence. Direct sequencing toward the segment of Hypervariable 1 (HV1) in the area of D-loop was conducted using 1 set of internal primer sequencing, i.e CR-foward (5'-tctatattccacatttctc-3') and CR-reverse (5'-gcgagcataaccaaatgg-3'). Sequencing was conducted using BigDye* Terminator version 3.1 Circle Sequensing kit (Applied Biosystem, USA) and the result of purification in the electrophoresis was sequenced in ABI 3730 XL automated capillary DNA sequencer (Applied Biosystems, USA).

Data Analysis. Fragmen D-loop DNAmt on the first 397 base pair length was used for analysis in this study. Sequence data was obtained after edited from the fragmen D-loop sequence. Sequence data was analysed with various kinds of computer software. Chromas was used for *viewing* and editing the sequence result. The ClustalX 1.83 was used for multiple alignment of sequences (Thompson *et al*. 1997; obtained from ftp://ftp-igbmc:u-strasbg.fr/pub/ClustalX). MacClade 4.0 was used to make the polymorphic sites (Maddison & Maddison 2000; available on http://ag.arizona.edu/macclade/macclade.html). Molecular evolutionary genetic analysis (MEGA) version 3.0 was used for phylogenetic and analysis of the molecular evolution (Kumar *et al*. 2004; available on http://www.megasoftware.net/), while Network analysis was used for illustrating haplotype diversity i.e. NETWORK 4.1.0.8 (Bandelt *et al*. 1999). MtDNA D-loop diversity indices, genetic differentiation, and mismatch distributions were determined using DnaSP version 4.0 (Rozas *et al*. 2003; available at http://www.ub.es/dnasp). Maternal genetic differentiation was further quantified using hierarchical analysis of molecular

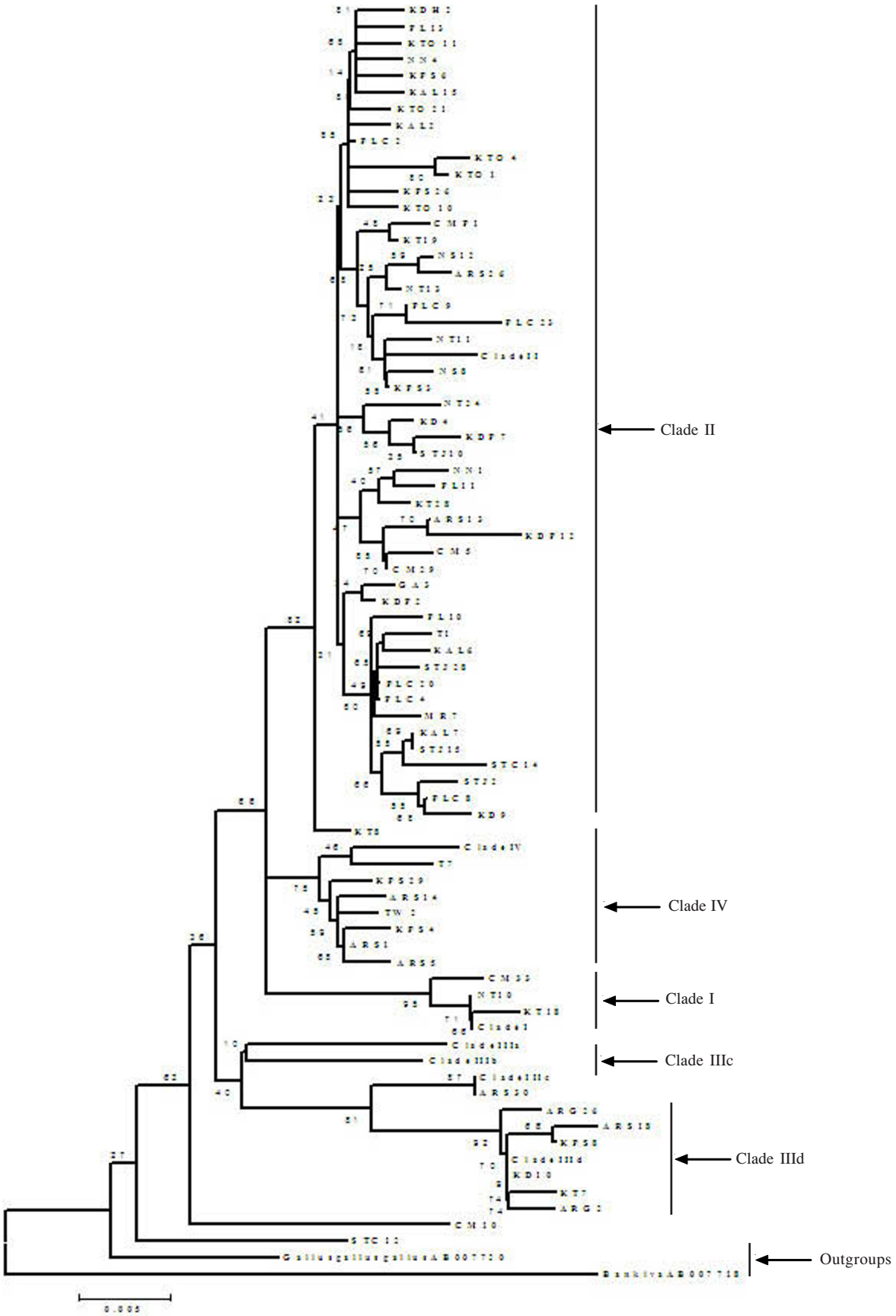

Figure 3. Phylogenic tree based on 69 haplotypes identified on Indonesian indigenous chicken, 2 haplotypes from the gen Gallus which sequence is taken from GenBank: *Gallus gallus gallus* (GenBank accession number AB007720) and *Gallus gallus bankiva* (GenBank accession number AB007718) and 7 Clade reference haplotypes (Clade I, II, IIIa, IIIb, IIIc, IIId, and IV). The numbers at the major nodes represent the percentage bootstrap values for interior branches after 1000 replications.

variance (AMOVA; Excoffier *et al*. 1992). Calculations were performed using computer software Arlequin version 3.01 (Excoffier 2006; available at http://anthro.unige.ch/arlequin).

Alignment of D-loop sequences was done to a referemce sequence from GenBank accession number AB0986688 (Komiyama *et al*. 2003). Three D-loop sequences of *Gallus* were included from GenBank with one of the haplotypes a domestic chicken, *Gallus gallus domesticus* (GenBank accession number AB009448). The other two, *Gallus gallus gallus* and *Gallus gallus bankiva* (GenBank accession numbers AB007720 and AB007718, respectively) were used as outgroups. To define the mitochondrial DNA lineages, seven clade reference haplotypes (Table 2): Clades I, II, IIIa, IIIb, IIIc, IIId, and IV were used in this analysis (Bjornstad *et al*. 2005 in Mobegi 2005). One for each clade were identified and aligned with our haplotypes.

RESULTS

Pattern of Mitochondrial DNA D-loop Variability. Four hundred and thirty four (434) of D-loop mtDNA sequences of Indonesian indigenous chickens were produced from the study. It came from chickens: Pelung, PL (n=47); Cemani, CM (n=34); Gaok, GA (n=7); Kedu, KD (n=37); Wareng, T (n=10); White Kedu, WK (n=21); Sentul, ST (n=42); Kate, KT (n=29); Arab Silver, ARS (n=30); Arab Golden, ARG (n=26); Merawang, MR (n=28); Kapas, KPS (n=21); Nunukan, N (n=55); Tolaki, KTO (n=17); and Kalosi, KAL (n=30).

Alignment of D-loop sequences was done to a referemce sequence from GenBank (accession number AB0986688) using ClustalX 1.8 software (Thomson *et al*. 1997). Sixty-nine haplotypes (sequence type) were identified with 54 polymorphic sites (*variable site*) (Figure 1), and the distribution of sequence variations in the first 397 nucleotides of D-loop HV1 region is seen in Figure 2. The complete alignment showed that there is higher variability between nucleotides 167 and 397 (around 94.5%), with only three polymorphic sites within the first 166 nucleotides giving very low sequence variation (only 5.5%).

Phylogenetic Analysis of Indonesian Indigenous Chickens. Phylogenetic analysis was done with mtDNA reference sequences representing seven reference clades (Clade I, II, IIIa, IIIb, IIIc, IIId, IV), as identified in a set of Asian samples (Bjornstad *et al*. 2005 unpublished *in* Mobegi 2005) (Table 2). The clade reference in this study is used to identify the phylogenic position of Indonesian indigenous chickens. As already mentioned in methods, three D-loop sequences of *Gallus* were included from GenBank with one of the haplotype a domestic chicken, *Gallus gallus domesticus* (GenBank accession number AB009448), and the other two, *Gallus gallus gallus* and *Gallus gallus bankiva* (GenBank accession numbers AB007720 and AB007718, respectively), as outgroups. The phylogenetic tree constructed for all 69 haplotypes is shown in Figure 3.

Figure 3 suggests that Indonesian chicken types were more genetically close to *Gallus gallus gallus* while their genetic distances with *Gallus gallus bankiva* were relatively further. To define the mtDNA lineages, one for each clade of reference haplotypes were identified and aligned with our haplotypes. All 69 haplotypes had fitted into five of seven clades observed in Asia. i.e. clade I, II, IIIc, IIId, and IV (Table 2), where clade I has 3 haplotypes, clade II has 50 haplotypes, clade IIIc has 1 haplotype, clade IIId has 6 haplotypes, and clade IV has 7 haplotypes. None of the samples in this study belonged to clade IIIa and IIIb. Most of the haplotypes were grouped into clade II which contributed 72% of the haplotypes.

From 69 haplotypes identified with variations at 54 sites, apparently 2 haplotypes (CM10 and STC 12, Figures 1 & 3) had separated or grouped into their own places and could not be grouped within the clade reference of indigenous chickens (clade I, II, IIIa, IIIb, IIIc, IIId, and IV).

Network Analysis of Indonesian Indigenous Chickens. Median-joining network analysis of the 69 haplotypes based on the variable characters of the complete alignment was conducted using computer program NETWORK 4.1.0.8 (Bandelt *et al*. 1999) and shown in Figure 4. Median joining network is made for describing a genetic variety of Indonesian indigenous chickens.

Indonesian indigenous chickens are different from other indigenous chickens in Asia, because Indonesian chickens dominantly enter clade II. By using the clade reference of indigenous chickens (clade I, II, IIIa, IIIb, IIIc, IIId, and IV) as presented in Table 2, data obtained in this study were combined with data obtained from International Livestock Research Institute (ILRI). The results (Figures 5 & 6) indicate there are 3 dominant clades in 3 different colours (yellow, green, and blue) and are represented by 3 countries: yellow for Indus valley (India), green for Yellow River (China), and blue for Indonesia.

Population Diversity of Indonesian Indigenous Chickens. The diversity indices calculated for all populations with all sequences are presented in Table 3. The highest number of haplotypes was found in populations of Sentul and Nunukan (H = 13) while the lowest number was detected in Gaok population (H = 3). The result shows that the diversity of haplotype of Indonesian indigenous chickens was between 0.45538-0.89832, where the lowest was Arab Golden chicken and the highest was Nunukan chicken. The average haplotype diversity over all populations was approximately 0.88045 for the 398 chicken D-loop sequences.

The nucleotide diversity, ð is a more suitable parameter than haplotype diversity to estimate the genetic diversity in populations. The former addresses both the frequency of haplotypes and nucleotide differences between haplotypes. The average nucleotide diversity in Indonesian indigenous chicken populations was estimated for the 398 D-loop sequences to be 0.00994 nucleotide substitutions per site. The diversity of nucleotide was between 0.00264-0.00828 where the highest was Wareng chicken and the lowest was Gaok chicken.

Analysis of Molecular Variance (AMOVA). Hierarchical AMOVA was done to give more insight on how genetic variation is distributed between individuals within populations, between populations within groups and between groups (Excoffier *et al*. 1992; Excoffier 2006). Analysis of molecular variance was based on Kimura-2-parameter distances. The

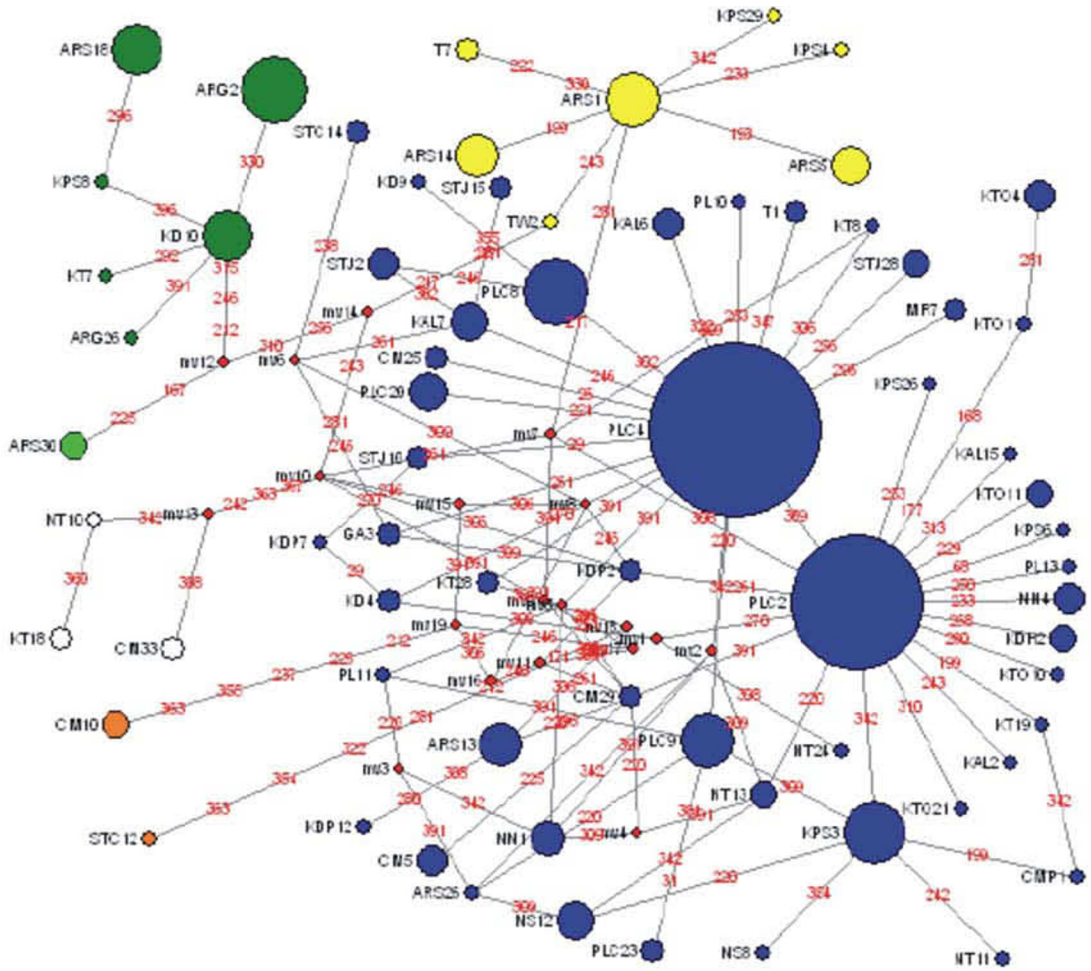

Figure 4. Median-joining network for 69 haplotypes of Indonesian indigenous chicken based on polymorphic site of the mitochondrial D-loop HV1 region. Area of each circle is proportional to the frequency of the corresponding haplotype. Different classes of haplotypes are distinguished by use of colour codes. White circles indicate clade I, blue circles denote clade II, yellow circles refer to clade IV, while colours red, light green, and dark green denote Clades IIIa, IIIc, and IIId respectively. The pink dots illustrate median vectors (mv), produced by the network software, representing putative intermediate haplotypes that have not been found or sampled.

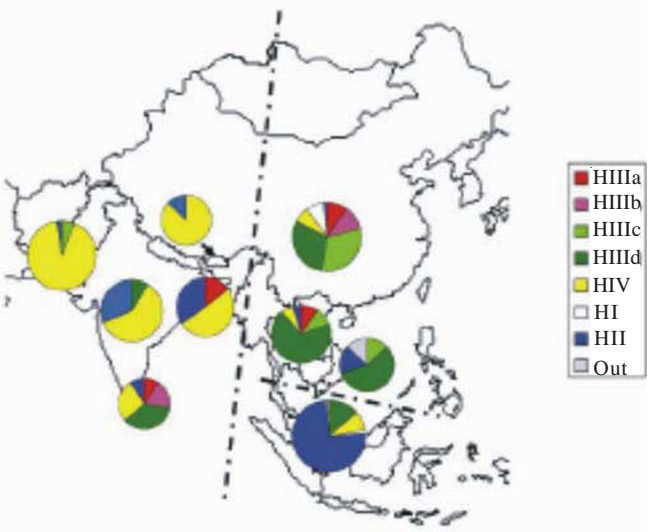

Figure 5. Three center domestication of chickens indicated by three dominant colours, green (Yellow River, China), yellow (Indus valley), and blue (Indonesia).

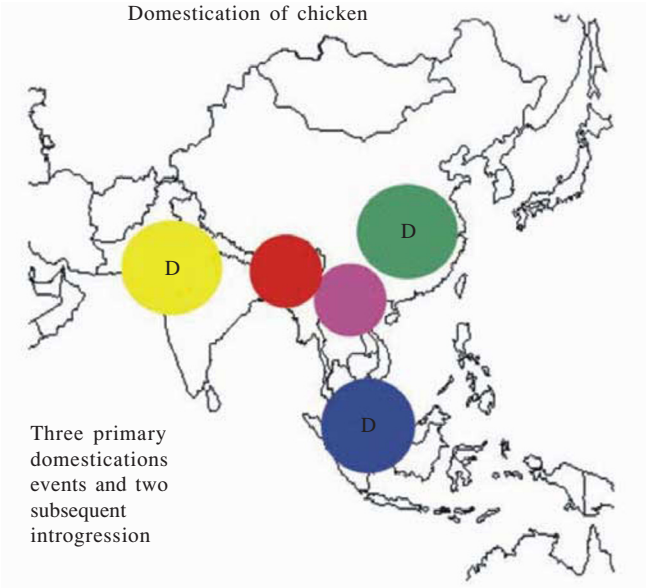

Figure 6. Center for indigenous chicken domestication.

calculations were performed based on 1,000 permutations using computer software Arlequin version 2,000 (Schneider *et al*. 2000). Results are presented in Table 4. The AMOVA result shows that genetic variation among chicken individuals within chicken population is 67.65%, while genetic variation among chicken breed population is 32.15%. The low genetic differentiation resulting from the geographical groups suggests that Indonesian indigenous chickens have not been subdivided across the regions hence this implies that breeding females may have been exchanged.

Table 2. Reference haplotypes from domestic chicken considered

Haplotype name	Code of haplotype	Sampling site
Clade I	AF128344*	China
Clade II	AB009436*	Lombok Island, Indonesia
Clade IIIa	FL17	Thailand
Clade IIIb	DW07	China
Clade IIIc	DW02	China
Clade IIId	DC15	China
Clade IV	PKD15	Pakistan

*GenBank accession numbers.

Genetic Distance Among Indigenous Chicken Populations. Genetic distance among chicken populations, such as seen in Table 5 below, was counted using DnaSP program. Data appearing in Table 5, i.e. the genetic distance which is far from Arab Gold chickens and chickens from another breeds, for example the genetic distance between Arab Gold with Gaok chicken is very far (0.88), followed by the genetic distance between Arab Gold and Kalosi chickens

Table 3. MtDNA diversity indices in Indonesian indigenous chicken populations based on 397 partial D-loop sequences

Island	Population	N	S	H	Hd	ð
Java	Cemani	34	18	9	0.74866	0.00815
Sumatra	Kapas	21	18	11	0.89524	0.01231
Java	Pelung	47	7	8	0.79556	0.00352
Sumatra	Arab Golden	26	13	5	0.45538	0.00389
Sumatra	Merawang	28	14	6	0.69048	0.00631
Sumatra	Arab Silver	30	21	10	0.87356	0.01068
Java	Kedu	37	21	11	0.82432	0.01111
Java	White Kedu	21	20	10	0.84286	0.00734
Java	Kate	29	19	10	0.72906	0.00642
Java	Gaok	7	2	3	0.76190	0.00264
Java	Sentul	42	24	13	0.85366	0.00740
Java	Wareng	10	8	5	0.86667	0.00828
Sulawesi	Tolaki	17	8	8	0.89706	0.00567
Sulawesi	Kalosi	30	11	9	0.87816	0.00512
East Kalimantan	Nunukan	55	21	13	0.89832	0.00697
Total		434	49	69	0.88045	0.00994

N: Number of sequence used, S: Number of segregation site, H: Number of haplotype, Hd: Diversity of haplotype, ð: Diversity of nucleotide. Genetic differentiation estimates: Hudson 2000, Snn (nearest neighbour statistic): 0.31780 PM test; P-value of Snn: 0.0000 *** (this indicates significant genetic differentiation). Gene Flow Estimates: Hudson, Slatkin and Maddison 1992. Fst: 0.33166 Nm: 1.01 (indicates that the exchange between populations is low).

Table 4. Analysis of molecular variance (AMOVA) between D-loop sequences haplotypes in Indonesian indigenous chicken

Source of variety	Free degree	Quadrat number	Variety component	Variety percentage (%)
Among population	15	339.102	0.79025Va	32.15
Within the population	419	698.637	1.66739Vb	67.65
Total	434	1037.739	2.45764	

Table 5. Pairwise F_{ST} (Hudson *et al*. 1992) between 15 chicken population based on D-loop sequence

	CM	KPS	PL	ARG	MR	ARS	KD	KDP	KT	GA	ST	TTW	KTO	KAL	NN
CM															
KPS	0.077														
PL	0.063	0.152													
ARG	0.772	0.623	0.861												
MR	0.045	0.063	0.034	0.791											
ARS	0.093	0.005	0.162	0.692	0.062										
KD	0.043	0.002	0.079	0.679	0.008	0.035									
KDP	0.011	0.045	0.018	0.775	0.022	0.054	0.003								
KT	0.028	0.099	0.021	0.802	0.004	0.113	0.039	0.006							
GA	0.178	0.232	0.138	0.877	0.14	0.264	0.147	0.138	0.062						
ST	0.034	0.071	0.035	0.775	0.002	0.089	0.017	0.014	0.027	0.161					
WAR	0.305	0.143	0.415	0.738	0.287	0.082	0.232	0.281	0.335	0.497	0.317				
KTO	0.15	0.114	0.229	0.813	0.189	0.137	0.141	0.131	0.205	0.396	0.169	0.333			
KAL	0.049	0.087	0.044	0.823	0.027	0.098	0.049	0.014	0.028	0.175	0.021	0.329	0.163		
NN	0.094	0.063	0.156	0.788	0.139	0.081	0.097	0.092	0.133	0.285	0.13	0.264	0.108	0.102	

Name of population CM: Cemani, KPS: Kapas, PL: Pelung, ARG: Arab Golden, MR: Merawang, ARS: Arab Silver, KD: Kedu, WK: White Kedu, KT: Kate, GA: Gaok, ST: Sentul, WAR: Wareng, KTO: Tolaki, KAL: Kalosi, NN: Nunukan.

which is 0.823. On the contrary, the closest genetic distance (0.002) occurs between Sentul and Merawang and also between Kedu and Kapas. Between Kedu and White Kedu there is a close genetic distance of 0.003.

DISCUSSION

Phylogenetic analysis of the 69 haplotypes defined in Indonesian indigenous chickens illustrates the evolutionary relationship as well as their genetic diversity. The phylogenetic tree of haplotypes (Figure 3) shows that Indonesian indigenous chickens falls into five clusters which represents five maternal lineages. Indonesian indigenous chickens are dominated by haplotype existing in clade II, covering 80.2% of the total chickens used in the study. Dominantly, Indonesian indigenous chickens are grouped into clade II. This is a frequent finding in Asia. It is higher than in Madagascar with around 75% of haplotypes, also in clade II (Mobegi 2005). Therefore, Indonesian indigenous chickens are very different from indigenous chickens of other Asian countries. But the question is why do Indonesian and Madagascar indigenous chickens dominate the group of clade II. This result is very interesting and needs further clarification to see whether there is a relationship between Madagascar and Indonesia, and how is the relationship? But according to history, the people of Madagascar originate from the Bugis ethnic group (Indonesia). There is a possibility that people from the Bugis ethnic group brought Indonesian indigenous chicken to Madagascar to be breed. It is believed that the cultural value of chickens accounts for its initial domestication and dispersion more than its importance today as a source of protein in the form of meat and eggs.

The network joining result (Figure 4) indicates the genetic variety of 434 individuals of Indonesian indigenous chickens. As constructed in the phylogeny tree (Figure 3), Indonesian indigenous chickens are grouped into 5 clades (clade I, II, IIIc, IIId, and IV) and have 5 different colors which is dominated by blue (clade II). Indonesian indigenous chickens are different from indigenous chickens of other Asian countries because they dominantly enter clade II. The number of haplotypes in clade II is 50, so there are also 50 circles of blue color. DNA sequence of haplotype PLC4 (Figure 4) has the highest frequency or the biggest blue circle. This haplotype PLC4 is related to the most number of other haplotypes. On the contrary, if a haplotype has a low frequency, it is indicated with a small circle. It is shown in the Figure 4 of median joining network that different clades are distinguished by color codes, i.e.: white: clade 1, blue: clade II, young green: clade IIIc, dark green: clade IIId, yellow: clade IV. The red point (small circle) shows the median vector (mv), while the figures among the nodes of haplotypes show the position of occurrence of the nucleotide mutation compared to sequence reference (GenBank accession number AB098668) (Komiyama *et al*. 2003).

Gaok chicken is an indigenous chicken breed, which was noticed to have the lowest level of diversity in nucleotide value. This might be because Gaok chickens were collected from one village in Bangkalan Distrtict. So the low diversity of nucleotide value may be connected to family, which has close relationships with Gaok chickens and this can be explained due to founder effects (e.g. a small effective population size of breeding females). A part from all those things, the attempt to conserve toward Indonesian indigenous chicken resource needs to be incited. Furthermore, this is also supported with data in Table 3, i.e. the genetic distance which is far from Arab Gold chickens, for example the genetic distance between Arab Gold with Gaok chicken is very far (0.88), followed by the genetic distance between Arab Gold and Kalosi chickens which is 0.823. On the contrary, the closest genetic distance (0.002) occurs between Sentul and Merawang and also between Kedu and Kapas. Between Kedu and White Kedu there is a close genetic distance of 0.003. The variety result as mentioned in the first sentence in this paragraph, identified the genetic distance of 15 chicken breeds. Information of the genetic distance is needed for conducting the crossing of chicken families with far genetic distance, to form a final stock of superior indigenous chicken in order to gain high hybreed vigor (effect *heterosys* positive). Inventory result of the Indonesian indigenous chicken family is found out to have specific phenotive variety which needs special attention from the regional government.

There is little doubt that successive domestication of various wild animals contributed greatly to the sustenance and cultural development of mankind. It involves adaptation of animals to environmental condition, therefore some changes in behaviour and physiology of the animal would be expected (Siegel & Dunnington 1990). As reported, these behavioral physiological changes associated with domestication is a must, however, these changes vary according to type of domestication whether it is toward meat or egg production. Archaeological discoveries in China indicate that chickens had been domesticated by 5400 B.C. and chickens from Harappan culture of the Indus Valley (2500-2100 B.C) may have been the main source for diffusion through the world (Crawford 1990a,b). Birds were first domesticated for cultural and entertainment purposes, until much later birds were utilized as a source for human food (Crawford 1990b). The center for chicken domestication in the world was known from 2 countries, i.e. Yellow River, Henan (China) and Indus valley area (India). From this study, it is concluded that Indonesian indigenous chickens are different from indigenous chickens of other Asian countries because it dominantly enters the clade II (Figures 3 & 4). Thus based on the composition of chicken clade in Asia (Figures 5), it shows that three large countries have very special composition of clades, i.e. Indus valley area is dominated by the population of clade IV; Yellow River, Henan is dominated by the population of clade IIId, and Indonesian area is dominated by clade II. The result is illustrated in Figures 5 and 6. It is concluded that Indonesia is one of three countries which are centers of chicken domestication.

Therefore, various other communities need to increase the use of biological resources through the improvement of productivity and immunity. The rich biological resource of Indonesian indigenous chickens which is spread throughout the area needs to be conserved by various communities. If

the preservation system is to be done by the community, it is highy needed to give them an understanding about preservation concept and procedures by competent institution. But what is more important for the preservation community is the economy and social use of the genetic source managed. However genetic resource conservation is still an expensive activity. Therefore, the conservation effort for some indigenous chicken breeds still needs to be followed up with more applicative study collaboration. The involvement of regions which has one or more breeds of indigenous chicken should be preserved in order to be supported by all parties.

This study has proved that mtDNA and more specifically D-loop HV1 segment is a powerful molecular tool in resolving phylogenetic relationships within a species and also understanding the genetic diversity. There is a high variability in the genetic diversity of Indonesian indigenous chickens with some populations showing high genetic diversities whereas other populations show low genetic diversities. But whether the low genetic diversity is due to loss of diversity should be cautiously interpreted because there were neither control samples nor information on the previous genetic diversity status for those populations.

ACKNOWLEDGEMENT

The study was supported by Competitive Grant-LIPI, Sub Program of Domestication (2005-2007). I greatly thank International Livestock Research Institute (ILRI) scientists Olivier Hanotte, Han Jianlin, and Victor Mobegi for all the discussions, from which I have learnt so much, and extend the same to all parties who have helped this study.

REFERENCES

Al-Nasser A *et al*. 2007. Overview of chicken taxonomy and domestication. *World's Poult Sci J* 63:285-400.

Bandelt HJ, Forster P, Rohl A. 1999. Median-joining networks for inferring intraspecific phylogenies. *Mol Biol Evol* 16:37-38.

Baumung ByR, Simianer H, Hoffmann I. 2004. Genetic diversity studies in farm animals-a survey. *J Anim Breed Genet* 121:361-373.

Brown WM, Prager EM, Wang A, Wilson AC. 1982. Mitochondrial DNA sequences of primates: Tempo and mode of evolution. *J Mol Evol* 18:225-239.

Bruford MW, Bradley DG, Luikart G. 2003. DNA markers reveal the complexity of livestock domestication. *Nat Rev Genet* 4:900-910.

Crawford RD. 1990a. Poultry Biology: Origin and History of Poultry Species. In: Crawford RD (ed). *Poultry Breeding and Genetics*. Amsterdam: Elsevier Science Publishing Company. p 1-42.

Crawford RD. 1990b. Poultry genetic resources: evolution, diversity, and conservation. In: Poultry Breeding and Genetics. Crawford (RD). Amsterdam: Elsevier Science Publishing Company. p 43-60.

Desjardins P, Morais R. 1990. Sequence and gene organization of the chicken mitochondrial genome. A novel gene order in higher vertebrates. *J Mol Biol* 212:599-634.

Excoffier L. 2006. Arlequin ver.3.01. *Computational and Molecular Population genetics lab. Zoological Institute*, University of Berne.

Excoffier L, Smouse P, Quattro J. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics* 131:479-491.

Fumihito A *et al*. 1994. One subspecies of the red junggle fowl (*Gallus gallus gallus*) suffices as the matriarchic ancestor of all indigenous breeds. *Proc Natl Acad Sci USA* 91:12505-12509.

Fumihito A *et al*. 1996. Monophyletic origin and unique dispersal patterns of indigenous fowls. *Proc Natl Acad Sci USA* 93:6792-6795.

Hashiguchi T, Nishida T, Hayashi Y, Maeda Y, Mansjoer SS. 1993. Blood protein polymorphisms of native and jungle fowls in Indonesia, Asian-Australas. *J Anim Sci* 6:27-35.

Ishida N *et al*. 1994. Polymorphic sequence in the D-loop region of equine mitochondrial DNA. *Animal Genetics* 25:215-221.

Jansen T *et al*. 2002. Mitochondrial DNA and the origins of domestic horse. *Proc Natl Acad Sci USA* 99:10905-10910.

Komiyama T, Ikeo K, Gojobori T. 2003. Where is the origin of the Japanese gamecocks? *Gene* 317:195-202.

Komiyama T, Ikeo K, Gojobori T. 2004a. The evolutionary origin of long-crowing chickens: its evolutionary relationship with fighting cocks disclosed by the mtDNA sequence analysis. *Gene* 333:91-99.

Komiyama T, Ikeo K, Tateno Y, Gojobori T. 2004b. Japanese domesticated chicken have been derived from Shamo traditional fighting cocks. *Molecular Phylogenetics Evolution* 33:6-21.

Kumar S, Tamura K, Nei M. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings Bioinformatics* 5:150-163.

Kusuma AS. 2002. Karakteristik sifat kuantitatif pada ayam kampung dan ayam merawang umur 5 dan 8 minggu yang diberi pakan komersial [Thesis]. Bogor: Institut Pertanian Bogor.

Liu ZG *et al*. 2004. Genetic variability of mtDNA sequences in Chinese Native Chicken Breeds. *Asian-Aust J Anim Sci* 17:903-909.

Loftus RT, MacHugh DE, Bradley DG, Sharp PM, Cunningham P. 1994. Evidence for two independent domestications of cattle. *Proc Natl Acad Sci USA* 91:2757-2761.

Luikart G *et al*. 2001. Multiple maternal origins and weak phylogeographic structure in domestic goats. *Proc Natl Acad Sci USA* 98:5927-5932.

Maddison DR, Maddison WP. 2000. MacClade 4: Analysis of phylogeny and character Evolution, 4.0. Sinauer Associates, Sunderland, MA.

Mansjoer I, Mansjoer SS, Sayuthi. 1989. Studi banding sifat biologis ayam Kampung, ayam Pelung, dan ayam Bangkok. *Buletin Penelitian IPB* 7:77-86.

Mansjoer SS *et al*. 1996. Studi morfometrik dan sifat-sifat genetic melalui polimorfisme protein pada ayam Sentul, Pelung, dan ayam Kampung. Laporan penelitian ARMP-Project, Kerjasama IPB dan Balitnak, Ciawi, Bogor.

Mobegi VA. 2005. Genetic characterization of African chicken using mitochondrial DNA D-loop sequences [Thesis]. Nairobi: Department of Biochemistry, Faculty of Medicine, University of Nairobi.

Nataamijaya AG. 2000. The native of chicken of Indonesia. Plasma Nutfah Bulletin Volume 6, No.1. Balai Penelitian dan Pengembangan Pertanian, Departemen Pertanian.

Nataamijaya AG, Jarmani SN, Sartika T. 1996. Konsep strategi penanganan pelestarian plasma nutfah pertanian secara ex-situ ternak ayam buras. Proyek Pemanfaatan dan Pelestarian Plasma Nutfah Pertanian, Bogor.

Niu D *et al*. 2002. The origin and genetic diversity of Chinese native chicken breeds. *Biochemical Genetics* 40:163-174.

Nishida T, Nozawa K, Hayashi Y, Hashiguchi T, Mansjoer SS. 1982. Body measurement and analysis of external genetics caharacters of Indonesian native fowl. The Origin and Phylogeny of Indonesian Native Livestock. III: 73-84.

Putra F. 1999. Pengaruh sifat bentuk jengger terhadap produksi telur ayam Kampung selama tiga bulan pertama [Skripsi]. Bogor: Jurusan Peternakan, Fakultas Pertanian, Universitas Juanda.

Rozas J, Sánchez-DelBarrio JC, Messegyer X, Rozas R. 2003. DnaSP, DNA polymorphism analyses by coalescent and other methods. *Bioinformatics* 19:2496-2497.

Sambrook J, Fritsch EF, Manias T. 1989. *Molecular Cloning*. A Laboratory Manual. Vol. 1-3. Second Edition. Cold Spring Harbour Laboratory Pr.

Sartika T, Duryadi D, Mansjoer SS, Gunawan B. 2000. Keragaman genetik ayam Kampung berdasarkan analisis penanda daerah D-loop Mitokondria DNA. *JITV* 5:100-106.

Sartika T, Iskandar S, Prasetyo LH, Takahashi H, Mitsuru M. 2004. Kekerabatan genetik ayam Kampung, Pelung, Sentul, dan Kedu Hitam dengan menggunakan penanda DNA mikrosatelit. I. Group pemetaan pada makrokromosom. *JITV* 9:81-86.

Savolainen P, Zhang YP, Lu J, Lundeberg J, Leitner T. 2002. Genetic evidence for an East Asian origin of domestic dogs. *Science* 298:1610-1613.

Schneider S, Roessli D, Excoffier L. 2000. Arlequin ver. 2.000: *A software for population genetics data analysis*. Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland.

Siegel PB, Dunnington EA. 1990. Behavioural genetics. In: Crawford RD (ed). *Poultry Breeding and Genetics*. Amsterdam: Elsevier Science Publ Comp. p 877-896.

Sunnucks P. 2000. Efficient genetic markers for population biology. *Trends Ecol Evol* 15:199-203.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25:4876-4882.

Quinn TW, Wilson AC. 1993. Sequence evolution in and around the mitochondrial control region in birds. *J Mol Evol* 37:417-425.